



Pregnanetriolone in paper-borne urine for neonatal screening for 21-hydroxylase deficiency: The place of urine in neonatal screening

José Ramón Alonso-Fernández^{1,2}

Laboratorio de Tría Neonatal en Galicia, Laboratorio de Metabolopatías, Departamento de Pediatría, Hospital Clínico (CHUS) e Universidade de Santiago de Compostela, Galicia, Spain

ARTICLE INFO

Article history:

Received 7 August 2016

Accepted 8 August 2016

Available online xxxx

Keywords:

Newborn screening

Congenital adrenal hyperplasia (CAH)

Urine sample on paper

Pregnanetriolone

ABSTRACT

The standard method of primary neonatal screening for congenital adrenal hyperplasia (CAH), determination of 17-hydroxyprogesterone (17OHP) in heelprick blood, is the object of recurrent controversy because of its poor diagnostic and economic efficiency. The superior ability of urinary pregnanetriolone levels to discriminate between infants with and without classical CAH has been known for some time, but has not hitherto been exploited for primary screening. Here we propose an economical neonatal CAH-screening system based on fluorimetric determination of the product of reaction between urinary pregnanetriolone and phosphoric acid.

© 2016 The Author. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Congenital adrenal hyperplasia (CAH) is an inherited metabolic disorder caused by autosomal recessive defects in the genes encoding enzymes involved in the biosynthesis of mineralocorticoids, glucocorticoids or sex steroids in the adrenal glands [1]. The most common such defect, accounting for 90–95% of CAH cases [2], affects *CYP21A2*, the gene encoding 21-hydroxylase, which transforms progesterone into deoxycorticosterone and 17 α -hydroxyprogesterone into 11-deoxycortisol. Unless otherwise stated, “CAH” hereinafter refers to CAH caused by 21-hydroxylase deficiency (21OHD). The reported frequency of this condition ranges from 1:28,000 in China to 1:280 among Yupik Eskimos, with an average of around 1:14,200 [3].

Depending on the specific mutation suffered, loss of 21-hydroxylase activity may be complete or only partial, and the degree of 21OHD correlates well, though by no means perfectly, with clinical phenotype [4]: total loss of activity typically results in the most severe form, salt-wasting CAH; mutations averaging 98% loss in simple virilizing CAH; and mutations causing 80–90% loss in mild, “non-classical” (late-onset) CAH. Non-classical CAH, which affects 0.1–0.2% of Caucasians [5–8], gives rise to no symptoms in early childhood, but later results in excess androgen levels and accelerated bone aging, and adolescent and adult females may suffer hirsutism, menstrual irregularity and infertility. In simple virilizing CAH (about a quarter of all “classical” CAH cases) the effects of excess androgen are already pronounced in early childhood; newborn girls may have ambiguous external genitalia. Salt-wasting CAH is a potentially life-threatening condition that, untreated, leads within 1–3 weeks of birth to acute adrenal crises, with poor weight gain, vomiting, diarrhoea, dehydration, failure to thrive, lethargy, hyponatraemia (due to 21OHD-induced aldosterone deficiency), hyperkalaemia, and shock.

Because of its high frequency and life-threatening potential, and because it can be treated effectively by corticoid replacement therapy, CAH is in many countries included among the inherited metabolic disorders screened for at birth. The standard primary screening method measures the 21-hydroxylase substrate 17-hydroxyprogesterone (17OHP) in the same kind of sample as is generally employed for other neonatal screening tests, blood obtained by heel prick and transported to the laboratory in sorbent paper. However, whatever the analytical technique used to determine 17OHP in these blood samples – the most widespread is dissociation-enhanced lanthanide fluoroimmunoassay DELFIA® (PerkinElmer Life Sciences-Wallac Oy, Turku, Finland) – this approach is bedeviled by the fact that 17OHP levels are generally high in unaffected newborns, especially if premature, sick or stressed [9–13]. In healthy newborns 17OHP levels rapidly fall, but their descent is often not completed before heelprick for neonatal screening (nowadays usually performed no later than the third day of life), which results in widely dispersed values [23]. Additionally, immunoassays suffer from a degree of cross-reactivity with sulphated steroids produced by the foetal and neonatal adrenal gland [9–14]. As a result of these two influences, there is considerable overlap between the 17OHP levels in heelprick samples of newborns with and without CAH: if a 17OHP screening threshold of adequate sensitivity is set, around 1% of all newborns test positive, and about 99% of these positive results will be false [9], making screening for CAH one of the least cost-effective of neonatal screening procedures [15–17].

For premature newborns the discrimination problem can be alleviated to some extent by using different 17OHP thresholds for different gestational age groups [2,18–20]. More generally, it has been reported that satisfactory sensitivity and positive predictive value can be achieved by subjecting positive-testing samples to a second-tier test in which liquid chromatography followed by tandem mass spectrometry (LC-MS/MS)

is employed to determine both 17OHP and other steroids (androstenedione, cortisol, 11-deoxycortisol, 21-deoxycortisol), and steroid ratios are used as the test criterion [9,11,21]. However, LC-MS/MS is an expensive procedure with low throughput (as is genotyping, another second-tier option [22]), and its application is costly, given the large number of first-tier false positives.

The basis for a radical possible solution to these problems was laid more than a decade ago when it was found that urinary pregnanetriolone differentiated perfectly between newborns with and without 21OHD [24–26]. The study of Homma et al. [24], was small on the non-21-OHD side (83 non-21OHD newborns with elevated blood 17OHP versus 50 term and 9 pre-term newborns with classical 21OHD), and the analytical method, gas chromatography with mass spectrometry, was not suitable for primary screening; but its findings have since been confirmed in a larger study [13], and there appears to be no reason why the inexpensive fluorimetric analytical procedure by means of which pregnane-triolone was first identified in patients' urine [27–32] cannot be readily adapted for reliable use in a screening programme. The major obstacle to the adoption of urinary pregnanetriolone as the analyte of choice for classical 21OHD screening seems to be the fact that the collection of urine samples – the basis of the pioneering neonatal screening programmes of Berry [33,34], Woolf [35–38] and others – has by many screening laboratories never been practised, and was discontinued long ago by most of those that did originally practise it.

The neonatal screening laboratory of Galicia (NW Spain) is one of the few that still collect urine samples from all newborns to screen for amino acid disorders, sugar disorders, mucopolysaccharidoses and oligosaccharidosis, and we are currently working to cover lysosomal storage disorders (glycosphingolipidoses, GM2 gangliosidoses, neutral glycosphingolipidoses, glycoproteinoses, mucopolipidoses, leukodystrophies and others), which are far easier to detect reliably using urine samples than in blood samples, in which the concentrations of marker substances are several times lower than in urine [38]. In the remainder of this article we describe the practical details of newborn urine sample collection, define a fluorimetry-based method of pregnanetriolone determination that we propose to validate, and outline a broader project for the evaluation of urinary-pregnanetriolone-based 21OHD screening in which we hope to enjoy the collaboration of other centres. We end with some final remarks on the future role of urine analysis in newborn screening.

2. Urine collection

In our screening system, urine samples, like heelprick blood, are taken on the third day of life [39], using a piece of Whatman 903 or Munktell TFN sorbent paper included in the screening kit (Fig. 1). Before

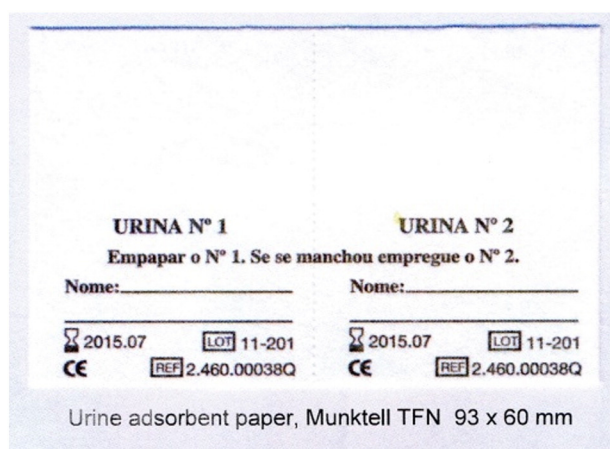


Fig. 1. Sorbent paper.

heelprick, half the paper is placed over the infant's genitals (which should be clean, with no cream, talc, oil or other product that might contaminate the urine) and is held in place by a diaper or napkin. Upon heelprick, the infant will generally urinate, after which the adsorbent paper can be removed and set apart to dry in a horizontal position on a support with which it makes minimal contact (e.g. a "bed of nails" made of pins stuck in cardboard, as in Fig. 2) [40]. In a maternity ward where samples are taken in a single session from all the infants born three days previously, it is of course necessary to write each infant's identity data on the sorbent paper before use; this should be done with a pencil so as to avoid contamination of the sample by ink. If upon heelprick the infant defecates as well as urinating, the sample should be discarded (even though the filter paper shows no visible faeces stain; it may bear fecal components eluted by the urine); in these cases, the other half of the filter paper is held in place with a diaper or napkin until the infant has urinated again. If the procedure takes place before discharge, the sample enters the laboratory on the day of collection; otherwise, if brought by hand or sent by mail.

3. Determination of pregnanetriolone

Sixteen discs 3 mm in diameter, or four 6 mm in diameter, are punched from the urine-bearing sorbent papers upon their arrival at our laboratory, and are stirred in the wells of 96-well microtitration plates containing 300 µL of water each (2 h, or 10 min if a 96-probe ultrasound device is used). The eluates thus prepared are currently employed to screen for the disorders mentioned in the Introduction by means of a battery of analyses that includes determination of creatinine for normalization [41,42]. Though subject to eventual optimization, the subsequent steps of the pregnanetriolone determination procedure that we propose to validate, adapted from Refs. 45 and 46 (see also Refs. 43 and 44), are as follows.

- Transfer 20 µL of eluate to a well of a black quartz microtitration plate (quartz to withstand heating, black to prevent interference between cells during fluorimetry – see below).

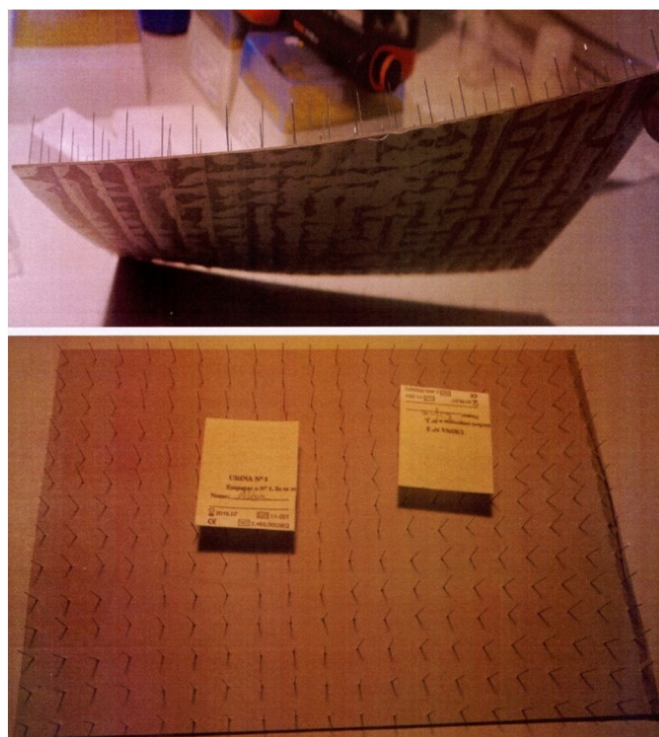


Fig. 2. Bed of nails.

- Add 4 μ L of conc. HCl per well, sandwich the plate between a 5-mm-thick sheet of red silicone below and a 5-mm-thick glass plate above, secure the sandwich with bulldog clips, and heat in an oven at 100 °C for 10 min so as to hydrolyse the sample and destroy steroids that would otherwise interfere with the determination of pregnanetriolone [45,46].
- Evaporate to dryness under a stream of nitrogen.
- Add 20 μ L of 85% phosphoric acid, sandwich the plate between silicone and glass as before, stir, heat for 5 min at 100 °C, and cool rapidly to room temperature.
- Read fluorescence at 530 nm under excitation at 440 nm [27], and obtain pregnanetriolone concentration from a calibration curve previously constructed using pure pregnanetriolone.

The excitation and emission wavelengths, 440 and 530 nm, will likely have to be modified somewhat, these wavelengths having been determined in the original studies [27] with fluorimeters in which wavelength was controlled only coarsely, using optical filters instead of the finer control methods employed in today's apparatus.

4. Evaluation of urinary-pregnanetriolone-based 21OHD screening

Evaluation of urinary pregnanetriolone as a basis for 21OHD screening will first require optimization and validation of the fluorimetric analytical method described above, with determination of its limits of detection and quantification, precision, accuracy, sensitivity, linearity, and vulnerability to interference. Although the hot acid treatment of the urine eluate will probably not eliminate all interference from other steroids, any remaining will doubtless derive, like pregnanetriolone, from elevated excretion due to CAH, and will accordingly not invalidate the test for screening purposes. Indeed, further adjustment of the excitation and emission wavelengths (in addition to the modifications mentioned in the previous paragraph) may allow such interferences to be exploited to good effect.

Once the analytical method has been optimized and validated, it will be necessary to evaluate its use for screening, determining diagnostic sensitivity and specificity, positive and negative predictive values, positive and negative likelihood ratios, diagnostic efficiency, and ROC curve [47]. Initially, the diagnostic threshold value specified by Homma et al. [24], 0.1 mg/(g creatinine), may be used, though as cases accumulate it may be found appropriate to establish a higher cutoff, given the probable contribution of fluorophores other than pregnanetriolone. Newborns testing positive would be followed up by quantification of serum 17OHP in serial blood samples (3:100 (v/v) propanol/heptane may be used to eliminate interfering steroids [48]), by measurement of 21-hydroxylase activity, and by any other tests deemed necessary, such as genotyping. No follow up of newborns with negative urinary pregnanetriolone tests would be necessary if, as in the authors' health system, the existence of undetected cases (false negatives) could readily be ascertained through on-line access to hospital records.

5. The role of urine samples in newborn screening

5.1. Concluding remarks

There is every reason to believe that the approach to CAH screening proposed here will prove to have better diagnostic characteristics - in particular superior positive predictive value and sensitivity - than the standard 17OHP-based approach, which is the object of recurrent controversy (quite recently in regard to its sensitivity [20] and its utility for pre-term infants [20]). The system proposed here would undoubtedly be more cost efficient than 17OHP-based screening, simply because it requires considerably less expensive consumables. If our expectations regarding its diagnostic efficiency are fulfilled, the major foreseeable

impediment to its widespread introduction would be unwillingness to collect urine samples as well as blood samples.

Urine samples, employed by the earliest phenylketonuria screening programmes [33–38,49], largely fell into disuse when the Guthrie test became the method of choice for phenylketonuria screening. We have argued elsewhere that their continued use is beneficial and advantageous in screening for a number of congenital disorders [38], and we have argued here that the possibility of including 21OHD in this group should be taken seriously and investigated. It is also well-known that lysosomal storage disorders are far easier to detect reliably using urine samples than blood samples, in which the concentrations of marker substances are several times lower than in urine [50]. In all these cases, urine analysis can or seems likely to provide a screening procedure - or at least a first-tier procedure - that is less expensive and no less diagnostically efficient than blood spot analysis. Is it not time that urine sampling be restored to the neonatal screening armamentarium?

Acknowledgements

We thank Ian Coleman for helping draft the manuscript; Drs. Paloma Cabanas and Amalia Andrade for helpful critical review; and Dr. A.J. Iglesias for photography.

References

- [1] T. Sánchez Bachega, B. Bilharinho De Mendoza, Hiperplasia Suprarrenal congénita, in: M. Pombo Arias (Ed.), *Tratado de Endocrinología Pediátrica*, 4.^a edición Interamericana, McGraw-Hill 2009, pp. 662–673 ISBN: 9788448163877 Depósito Legal: M-28168-2009. Capítulo 56.
- [2] P.W. Speiser, Editorial: improving neonatal screening for congenital adrenal hyperplasia, *J. Clin. Endocrinol. Metab.* 89 (8) (2004) 3685–3686.
- [3] S. Pang, W. Murphey, L.S. Levine, D.A. Spence, A. Leon, S. LaFranchi, A.S. Surve, M.I. New, A pilot newborn screening for congenital adrenal hyperplasia in Alaska, *J. Clin. Endocrinol. Metab.* 55 (3) (1982) 413–420.
- [4] P.W. Speiser, J. Dupont, D. Zhu, J. Serrat, M. Buegeleisen, M.T. Tusie-Luna, M. Lesser, M.I. New, P.C. White, Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency, *J. Clin. Invest.* 90 (1992) 584–595.
- [5] Joint LWPES/ESPE CAH Working Group, CONSENSUS. Consensus statement on 21-hydroxylase deficiency from the Lawson Wilkins pediatric Endocrine Society and the European Society for Paediatric Endocrinology, *J. Clin. Endocrinol. Metab.* 87 (9) (2002) 4048–4053.
- [6] P.W. Speiser, R. Azziz, L.S. Baskin, L. Ghizzoni, T.W. Hensle, D.P. Merke, H.F.L. Meyer-Bahlburg, W.L. Miller, V.M. Montori, S.E. Oberfield, M. Ritzen, P.C. White, Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline, *J. Clin. Endocrinol. Metab.* 95 (9) (2010) 4133–4160.
- [7] P.W. Speiser, R. Azziz, L.S. Baskin, L. Ghizzoni, T.W. Hensle, D.P. Merke, H.F.L. Meyer-Bahlburg, W.L. Miller, V.M. Montori, S.E. Oberfield, M. Ritzen, P.C. White, A summary of the Endocrine Society clinical practice guidelines on congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency, *Int. J. Pediatr. Endocrinol.* (2010), 494173, <http://dx.doi.org/10.1155/2010/494173> (5 pages).
- [8] R.J. Auchus, et al., Guidelines for the development of comprehensive care centers for congenital adrenal hyperplasia: guidance from the CARES Foundation initiative, *Int. J. Pediatr. Endocrinol.* (2010), 275213, <http://dx.doi.org/10.1155/2010/275213> (17 pages).
- [9] K. Sarafoglou, A. Gaviglio, A. Hietala, G. Frogner, K. Banks, M. McCann, W. Thomas, Comparison of newborn screening protocols for congenital adrenal hyperplasia in preterm infants, *J. Pediatr.* 164 (2014) 1136–1140.
- [10] P.R. White, Optimizing newborn screening for congenital adrenal hyperplasia, *J. Pediatr.* 163 (1) (2013) 11–12.
- [11] C.L. Chan, K. McFann, L. Taylor, D. Wright, P.S. Zeitler, J.M. Barker, Congenital adrenal hyperplasia and the second newborn screen, *J. Pediatr.* 163 (1) (2013) 109–113.
- [12] B.A. Tarini, The value of time in assessing the effectiveness of newborn screening for congenital adrenal hyperplasia, *JAMA Pediatr.* 168 (6) (2014) 515–516.
- [13] C. Kamrath, M.F. Hartmann, C. Boettcher, K.-P. Zimmer, S.A. Wudy, Diagnosis of 21-hydroxylase deficiency by urinary metabolite ratios using gas chromatography-mass spectrometry analysis: reference values for neonates and infants, *J. Steroid Biochem. Mol. Biol.* 156 (2016) 10–16.
- [14] M. Leal Nascimento, A.N. Baptista Cristiano, T. de Campos, M. Ohira, E. Cechinel, G. Simoni, J. van de Sande Lee, R.M. Muller Linhares, P.C. Alves da Silva, Then-year evaluation of a neonatal screening program for congenital adrenal hyperplasia, *Arq. Bras. Endocrinol. Metabol.* 58 (7) (2014) 765–771.
- [15] B.K. Yoo, S.D. Grosse, The cost effectiveness of screening newborns for congenital adrenal hyperplasia, *Public Health Genomics* 12 (2009) 67–72.
- [16] A. Alzares, S. Alolayan, H. Almutairi, S.S. Alqahtani, B. Rittenhouse, Re-evaluating the cost-effectiveness of screening for congenital adrenal hyperplasia (CAH): the sensitivity to choice of distributions in probabilistic sensitivity analysis (PSAs), *Value Health* 18 (7) (2015) A668, <http://dx.doi.org/10.1016/j.jval.2015.09.2439>.

- [17] A. Alotaibi, M. Alsheikh, S.R. Mahida, B. Rittenhouse, Exploring cost-effectiveness heterogeneity by state: using state-level vs. national disease incidence in newborn screening (NBS) cost-effectiveness analysis of congenital adrenal hyperplasia (CAH), *Value Health* 18 (7) (2015) A755, <http://dx.doi.org/10.1016/j.jval.2015.09.2932>.
- [18] H. Van der Kamp, C.G.M. Oudshoorn, B. Elvers, M. Van Baarle, B.J. Otten, J.M. Wit, P.H. Verkerk, Cut off levels of 17- α -hydroxyprogesterone in neonatal screening for congenital adrenal hyperplasia should be based on gestational age rather than on birth weight, *J. Clin. Endocrinol. Metab.* 90 (7) (2005) 3904–3690.
- [19] H.J. Van Der Kamp, J.M. Wit, Neonatal screening for congenital adrenal hyperplasia, *Eur. J. Endocrinol.* 151 (2004) 471–475.
- [20] S. Gidlöf, A. Wedell, C. Guthenberg, U. von Döbeln, A. Nordenström, Nationwide neonatal screening for congenital adrenal hyperplasia in Sweden. A 26-year longitudinal prospective population-based study, *JAMA Pediatr.* 168 (6) (2014) 567–574.
- [21] C.Z. Minutti, J.M. Lacey, M.J. Magera, S.H. Hahn, M. McCann, A. Schulze, D. Cheillan, C. Dorche, D.H. Chace, J.F. Lymp, D. Zimmerman, P. Rinaldo, D. Matern, Steroid profiling by tandem mass spectrometry improves the positive predictive value of newborn screening for congenital adrenal hyperplasia, *J. Clin. Endocrinol. Metab.* 89 (8) (2004) 3673–3693.
- [22] S. Kösel, S. Burggraf, R. Fingerhut, H.G. Dörr, A.A. Roscher, B. Olgemoller, Rapid second-tier molecular genetic analysis for congenital adrenal hyperplasia attributable to steroid 21-hydroxylase deficiency, *Clin. Chem.* 51 (2) (2005) 298–304.
- [23] F. Schreiner, M. Tozakidou, R. Maslak, U. Holtkamp, M. Peter, B. Gohlke, J. Woelfe, Functional glucocorticoid receptor gene variants do not underlie the high variability of 17-hydroxyprogesterone screening values in healthy newborns, *Eur. J. Endocrinol.* 160 (4) (2009) 667–673.
- [24] K. Homma, T. Hasegawa, E. Takeshita, K. Watanabe, M. Anzo, T. Toyoura, K. Jinno, T. Ohashi, T. Hamajima, Y. Takahashi, T. Takahashi, N. Matsuo, Elevated urine pregnanetriolone definitively establishes the diagnosis of classical 21-hydroxylase deficiency in term and preterm neonates, *J. Clin. Endocrinol. Metab.* 89 (12) (2004) 6087–6091.
- [25] M.P. Caulfield, T. Lynn, M.E. Gottschalk, K.I. Jones, N.F. Taylor, E.M. Malunowicz, C.H.L. Shackleton, R.E. Reitz, D.A. Fisher, The diagnosis of congenital adrenal hyperplasia in the newborn by gas chromatography/mass spectrometry analysis of random urine specimens, *J. Clin. Endocrinol. Metab.* 87 (8) (2002) 3682–3690.
- [26] K. Homma, T. Hasegawa, M. Masumoto, E. Takeshita, K. Watanabe, H. Chiva, T. Kurosawa, T. Takahashi, N. Matsuo, Reference values for urinary steroids in Japanese newborn infants: gas chromatography/mass spectrometry in selected ion monitoring, *Endocr. J.* 50 (6) (2003) 783–792.
- [27] B. Zondek, M. Finkelstein, Quantitative estimation of 3 α ,17,20 α -pregnanetriol-11-one in urine as an aid in the diagnosis of pseudohermaphroditic conditions, *Acta Endocrinol.* 17 (1–4) (1954) 452–459.
- [28] S. Goldberg, M. Finkelstein, Studies in steroids excretion in patients with a virilizing syndrome, *Bull. Res. Counc. Isr.* 5A (1956) 310.
- [29] F.J. Thomas, A.W. Steinbeck, Semiquantitative estimation of urinary pregnanetriol, pregnanetriolone and tetrahydro S in the investigation of adrenocortical function, *Acta Endocrinol.* 60 (4) (1969) 645–656.
- [30] M. Finkelstein, S. Goldberg, A test for qualitative estimation of pregnane-3- α ,20- α -triol-11-one in urine, and its significance in adrenal disturbances, *J. Clin. Endocrinol. Metab.* 17 (1957) 1063–1070.
- [31] J. Morato Monaro, I. Matos Spremolla, J. Maggiolo, F. Mane, J.M. Cerviño, Assay of pregnanetriolone in urine by paper chromatography, its use in several endocrine diseases, *An. Fac. Med. Montev.* 49 (5–6) (1964) 509–514.
- [32] M. Finkelstein, R.I. Cox, Method for simultaneous estimation of pregnane-3 α ,17 α ,20 α -triol and pregnane-3 α ,17 α ,20 α -triol-11-one in urine, *Proc. Exp. Biol. Med.* 95 (2) (1957) 297–300.
- [33] H.K. Berry, B. Sutherland, G.M. Guest, J. Warkain, Simple method for detection of phenylketonuria, *JAMA* 167 (1958) 2189–2190.
- [34] H.K. Berry, Procedures for testing urine specimens dried on filter paper, *Clin. Chem.* 5 (1959) 603–608.
- [35] L.I. Woolf, Large-Scale Screening for Metabolic Disease in the Newborn in Great Britain, in: J.A. Anderson, K.F. Swaiman (Eds.), *Phenylketonuria and Allied Metabolic Disease*, Children's Bureau, Washington 1967, pp. 50–61.
- [36] J.R. Alonso-Fernández, C. Colón, The contributions of Louis I. Woolf to the treatment, early diagnosis and understanding of phenylketonuria, *J. Med. Screen.* 16 (2009) 205–211.
- [37] J.R. Alonso-Fernández, C. Colón, Newborn screening in Spain with particular reference to Galicia: echoes of Louis I Woolf, *Mol. Genet. Metab.* 101 (2010) 95–98.
- [38] J.R. Alonso-Fernández, C. Colón, Aportaciones de Louis I. Woolf Al Tratamiento Y diagnóstico Precoz de La Fenilcetonuria Y Otros Errores congénitos Del Metabolismo. Los Comienzos de La tria Neonatal en España, Con Referencia Al Programa de Galicia, ResearchGate, 2015, <http://dx.doi.org/10.13140/RG.2.1.5044.1049>.
- [39] Actualización do Programa Galego Para a detección Precoz de Enfermidades endócrinas e metabólicas en Periodo Neonatal, Resultados 1995–2006, Xunta de Galicia. Consellería de Sanidade. Dirección Xeral de Saúde Pública, 2008 (35 pp).
- [40] M. García, J.R. Alonso-Fernandez, A. Escarpa, Copper nanowires immobilized on the boards of microfluidic chips for the rapid and simultaneous diagnosis of galactosemia diseases in newborn urine samples, *Anal. Chem.* 85 (2013) 9116–9125.
- [41] J.R. Alonso-Fernandez, J. Fidalgo, C. Colon, Neonatal screening for mucopolisaccharidoses by determining glycosaminoglycans in the eluate of urine impregnated in paper. Preliminary results of an improved DMB-based procedure, *J. Clin. Lab. Anal.* 24 (3) (2010) 149–153.
- [42] J.R. Alonso-Fernandez, A. Sedes, C. Colon, J. Fidalgo, Newborn screening for oligosaccharides. SSIEM 2007 annual Symposium Society for the Study of inborn errors of metabolism, September 4th–7th, 2007, J. Inher. Metab. Dis. 30 (Suppl. 1) (2007) 8.
- [43] M. Finkelstein, Pregnanetriolone, an Abnormal Urinary Steroid, in: R.I. Dorfman (Ed.), *Methods in Hormone Research*, vol. 1, Academic Press, New York 1962, pp. 169–197.
- [44] M. Rubin, Fluorimetry and phosphorimetry in clinical chemistry, *Adv. Clin. Chem.* 13 (1970) 16–269.
- [45] G.F. Marrian, S.M. Atherden, Urinary adrenocortical steroids: II. Formaldehydogenic substances in extracts of hot acid-hydrolysed urine, *Acta Endocrinol.* 13 (1953) 265–285.
- [46] B. Zondek, M.A. Finkelstein, Neutral Fluorogenic substance in human urine and its increased excretion in pregnancy and pseudohermaphroditism, *Acta Endocrinol.* 11 (1952) 297–305.
- [47] N.I. Wald, J. Bestwick, Is the area under an ROC curve a valid measure of the performance of a screening or diagnostic test? *J. Med. Screen.* 21 (1) (2014) 51–56.
- [48] M.A. Andrade Olivé, Estudio de la utilidad diagnóstica de la determinación de IGFBP-3 en niños de talla baja, Influencia de la Edad, Sexo, Estado Puberal y Estado Nutricional en los Niveles de IGF-I e IGFBP-3 en una población pediátrica Gallega, Universidade de Vigo, 1998 Tesis de Doctorado.
- [49] J.M. Fraga, J.R. Alonso-Fernández, M.D. Bóveda, J.A. Cocho, M. Bravo, J. Peña, The Organization and Methods of Neonatal and Metabolic Screening in the Regional Centre of Galicia (Spain), in: Therrell BL Jr. (Ed.), *Advances in Neonatal Screening*, Excerpta Medica 1987, pp. 481–483.
- [50] P.J.I. Meikle, E. Rianeri, H. Simonsen, T. Rozaklis, R. SL, P.D. Whitfield, M. Fuller, E. Christensen, F. Skovby, J.J. Hopwood, Newborn screening for lysosomal storage disorders: clinical evaluation of a two-tier strategy, *Pediatrics* 114 (2004) 909–916.